Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor

Takeshi Nakahashi, Hironobu Fujimura, C. Anthony Altar, Jess Li, Jun-ichi Kambayashi, Narendra N. Tandon, Bing Sun*

Maryland Research Laboratories, Otsuka America Pharmaceutical, Inc., 9900 Medical Center Drive, Rockville, MD 20850, USA

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Abstract Brain-derived neurotrophic factor (BDNF) is an abundant neurotrophin in brain and peripheral nerves, where it affects neural development, survival and repair after injury. BDNF has been detected in rat and human blood, but the source of circulating BDNF is not established. BDNF messenger and peptide were detected in cultured cells and in the culture medium of human umbilical vein endothelial cells. The expression of BDNF was up-regulated by elevation of intracellular cAMP and down-regulated by Ca^{2+} ionophore, bovine brain extract and laminar fluid shear stress. These results suggest that vascular endothelial cells may contribute to circulating BDNF.

Key words: Neurotrophin; Human umbilical vein endothelial cell; cAMP; Platelet

1. Introduction

Neurotrophins form a large family of dimeric polypeptides that include nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, NT-6 and NT-7 [1–3]. They are known to promote the growth, survival and differentiation of developing neurons in the central and peripheral nervous system [4]. BDNF also supports the maintenance and regeneration of neurons in the central nervous system (CNS), and enhances the short-term survival of axotomized mammalian retinal ganglion cells [5]. Neurotrophins are also widely distributed in the peripheral nervous system [6], and can promote the survival and neurite outgrowth of placodal and neural crest-derived sensory neurons [7]. BDNF, given peripherally, accelerates the regenerative sprouting of injured adult spinal motoneurons and axotomized retinal ganglion cells [8]. Therefore, BDNF may be involved in peripheral sensory and motor neuron regeneration at the site of nerve injury.

Although BDNF is highly concentrated in the nervous system, it is also present in human and rat plasma (109 and 150 pg/ml, respectively) and is much more concentrated in the serum [9]. However, the source of circulating BDNF remains unknown and several candidate cell types are considered in this report. Human platelets, which contain BDNF mRNA and release BDNF protein [9,10], might be a source of circulating BDNF. It is established that platelets have limited protein synthesis capacity, however, many substances (e.g. von Willebrand factor, fibrinogen, albumin and serotonin) that are stored and released by platelets are sequestered from the blood. Megakaryocytes are the progenitor cells for platelets. Thus, BDNF could be made by megakaryocytes and passed to the platelets as they are pinched off of the megakaryocytes. Alternatively, our recent demonstration of BDNF binding to platelets suggests that BDNF may be sequestered into platelets rather than synthesized in them [11]. The pituitary gland, where the anterior and neurointermediate lobes contain BDNF mRNA [12], could also be a source of circulating BDNF. However, BDNF levels in rat platelets and sera were not altered following long-term hypophysectomy (unpublished observation). Several neurotrophins including BDNF are expressed in non-neuronal organ systems including developing heart [13], vascular smooth muscle cells [14], developing intestine [15] and spleen [16], besides platelets. Recently, activated human T cells, B cells and monocytes have been found to release BDNF in vitro and in the brain regions of inflammatory lesions [17]. In previous studies with platelets, the expression of BDNF was detected with the polymerase chain reaction (PCR), a very sensitive method. In the present study, we investigated and quantified the expression of BDNF in vascular endothelial cells, megakaryocytic cells and platelets. It is demonstrated that BDNF is synthesized and secreted by human umbilical vein endothelial cells (HUVECs), and that the BDNF expression in HUVECs is regulated by intracellular cyclic adenosine monophosphate (cAMP), bovine brain extract (BBE) and laminar fluid shear stress.

2. Materials and methods

2.1. Cell culture and platelet preparation

HUVECs were obtained by collagenase (Sigma, St. Louis, MO, USA) digestion of human umbilical vein as described [18]. The cells were cultured at 37°C in Medium 199 with Earle's salts (Life Technologies, Rockville, MD, USA), containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.015% BBE and 0.01% heparin under humidified 5% CO_2/95% air. BBE was kindly provided by M. Fong and prepared according to a published method [19]. Briefly, 740 g of bovine hypothalamus was homogenized in 1 l of 0.15 M NaCl at 4°C for 4 min followed by stirring at 4°C for 2 h and subsequent centrifugation at $13\,800 \times g$ for 40 min. The supernatant was collected and stirred for approximately 1 h at 4°C after adding streptomycin sulfate (Sigma) to a final concentration of 0.5% (pH 7.0). The mixture was centrifuged and the supernatant was filtered, lyophilized and stored at −20°C for future use. The second to the eighth passaged HUVECs were used in the studies. The cells were sub-cultured using trypsin–EDTA, and seeded onto gelatin (0.2%)-coated petri dishes (35 mm in diameter) or 6 well plates. Isolated human coronary artery smooth muscle cells (HCASMCs) were purchased from Clonetics (Walkersville, MD, USA) and maintained in the medium recommended by the vender. Megakaryocytic cell lines

*Corresponding author. Fax: (1)-301-424 9054.
E-mail: bings@mrl.oapi.com

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(Meg-01, K562) were purchased from ATCC and cultured in F12K (Life Technologies, Rockville, MD, USA) containing 10% FCS.

Blood was drawn from healthy volunteers and mixed with 1/10 volume of 3.8% sodium citrate, followed by centrifugation at 150×*g* for 20 min. Supernatant (platelet rich plasma, PRP) was collected without disturbing buffy coat and packed red blood cells. Contaminating leukocytes and red blood cells were removed by an additional centrifugation for 10 min at 150×*g*. The pH of resultant PRP was lowered to 6.5 with citric acid (4 μl/ml from 1 M stock) prior to sedimentation of platelets at 800×*g* for 15 min. The pellet was used for RNA extraction.

2.2. Measurement of BDNF with enzyme-linked immunosorbent assay (ELISA)

When the cells reached confluence, culture medium was replaced with 2 ml of new medium after washing the cells three times with sterile phosphate-buffered saline (PBS, pH 7.4). At 12, 24, 36 and 48 h of incubation, 200 μl culture medium was collected and frozen at −70°C. To study the regulation of BDNF expression, confluent HUVECs were changed to new medium without BBE. Then, BBE (0.015%), interleukin (IL)-1β (10 ng/ml), transforming growth factor (TGF) β (10 ng/ml), forskolin (1 and 10 μM), thrombin (1 and 4 U/ml), histamine (10 μM), cycloheximide (CHX, 1 ng/ml), A23187 (1 μM) and 8-bromo-cAMP (500 μM; all above from Sigma) were added at time 0. At the end of experiments, the supernatants were collected and stored at −70°C until measurement. The cells were washed three times with PBS before being lysed in 200 μl of 1% Triton X-100 in 50 mM Tris–HCl (pH 7.5) plus phenylmethylsulfonyl fluoride (1 mM), *N*-ethylmaleimide (1 mM), leupeptin (2 μg/ml), NaF (1 mM), benzamidine (1 mM) and sodium vanadate (Na_3VO_4, 1 mM; all from Sigma). After incubation on ice for 1 h, the lysates were centrifuged at 12 000×*g* for 30 min. The BDNF in cell culture medium and cell lysates was measured by a commercial ELISA kit (Promega). The detection limit (sensitivity) of the ELISA is 15.6 pg/ml, with a coefficient of variance of 8.8% at a mean concentration of 28.6 pg/ml BDNF, according to the manufacturer's brochure. It was noticed that the ELISA measurement of BDNF was affected by the dilution of samples. In the early experiments, we measured BDNF in the samples without dilution. The calculated values are lower than that obtained later when we diluted the samples 1:1 with the ELISA dilution buffer. To test whether BDNF has any autocrine/paracrine effect on BDNF expression in HUVECs, recombinant human BDNF (200 pg/ml) was added to the medium without BBE; and neutralizing chicken polyclonal antibodies against human BDNF were added to the medium containing BBE (both from Promega, Madison, WI, USA). For this experiment, BDNF expression was measured by reverse transcription and PCR (RT-PCR) (Section 2.3). Data are described as the mean ± S.D. Student's *t*-test was used for determining statistical significance.

2.3. RT-PCR

Total RNA from HUVECs, platelets and other cells was extracted using RNA Stat-60 kit (Tel-test, Friendswood, TX, USA). The purity of RNA was determined by spectrophotometric measurements at 260 and 280 nm. One μg total RNA was reverse-transcribed into complementary DNA (cDNA) in 20 μl solution, using a Supertranscriptase II kit (Life Technologies). One μl RT reactions were used for each PCR amplification with a pair of specific primers for BDNF (forward 5′-AGAGTGATGACCATCCTTTTCC-3′ and reverse 5′-GCAGCCTTCTTTTGTGTAACC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward 5′-ATCAATGGAAATCCCATCACC-3′ and reverse 5′-TCTCTTCCTCTTGTGCTCTTGC-3′). PCR amplification was performed using a Perkin-Elmer 9700 thermal cycler for cycles as indicated, consisting of denaturation at 95°C for 1 min, annealing at the indicated temperature for 30 s and synthesis at 72°C for 1 min. The annealing temperatures and number of cycles for BDNF or GAPDH were 55°C or 55°C and 32 or 28 cycles, respectively. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The sequence of amplified DNA fragments was confirmed by sub-cloning them into plasmid vector pCR-II (Invitrogen, Carlsbad, CA, USA) followed by automatic DNA sequencing using an ABI 377 sequencer.

2.4. Northern blot analysis

The cDNA fragment of BDNF obtained by RT-PCR from HUVECs was labeled with [^{32}P]dCTP by nick translation and used as probe. Total RNA (20 μg) was extracted from pooled, fresh human platelets, cultured HUVECs, HCASMCs, Meg-01 and K562 cells, and run on a 1% denatured formaldehyde agarose gel. After blotting and fixing on a nylon membrane (Immobilon-Ny$^+$, Millipore, Medford, MA, USA), the membrane was incubated with the probe and allowed to hybridize at 42°C overnight. The membrane was then washed three times with 1×SSC plus 0.1% sodium dodecyl sulfate (SDS) at 65°C. Prior to exposing to X-ray film, the membrane was washed once with 0.2×SSC containing 0.1% SDS.

2.5. Laminar fluid shear stress loading

The shear loading system was modified from the cone-plate viscometer device, which can load well-defined laminar fluid shear stress to the cultured HUVECs [20]. The shear stress device consists of a cone that rotates above a stationary base plate containing the cultured HUVECs. The base plate is made from a 35 mm diameter gelatin-coated polystyrene dish (Corning, Marlboro, MD, USA). The cone makes an angle of 0.5° with the base plate; and its rotation is controlled by a motor with adjustable speed. The shear stress device was operated in a CO_2 incubator. Confluent HUVECs in a 35 mm dish were washed three times with PBS. 1.15 ml of culture medium was added. The distance between the cone and the culture dish was adjusted to 50 μm. The HUVECs were then exposed to shear stress (24 dyne/cm^2) for 24 h, after 30 min of pre-incubation in the incubator. Control samples without loading shear stress were obtained by the same procedure but without rotation.

3. Results

3.1. BDNF production by endothelial cells

BDNF in the HUVEC medium was measured by ELISA. As shown in Fig. 1, the concentration of BDNF in the medium after 48 h of incubation without BBE ranged from 15.3 to 251 pg/ml with an average of 135 ± 67.2 pg/ml ($n = 18$). BDNF in the cell lysates was 52.6 ± 7.0 pg/ml ($n = 3$). BDNF in the culture medium was lower when the cells were cultured with BBE (range from undetectable to 57 pg/ml with an average of 16.8 ± 14.8 pg/ml, $P < 0.01$). BDNF in the cell lysates was also significantly lower (27.6 ± 13.6 pg/ml, $n = 3$) than in cells cultured with BBE ($P < 0.05$). BDNF was also detected in the culture media of two other primary endothelial cell cultures of human coronary artery and bovine pulmonary artery, with concentrations ranging from 22 to 373 pg/ml (data not shown). Comparison of the BDNF content inside the HUVECs (10.5 pg on average in a given petri dish at 48 h) and the 270 pg BDNF in the culture medium in the absence of BBE suggests that most of the BDNF synthesized in the

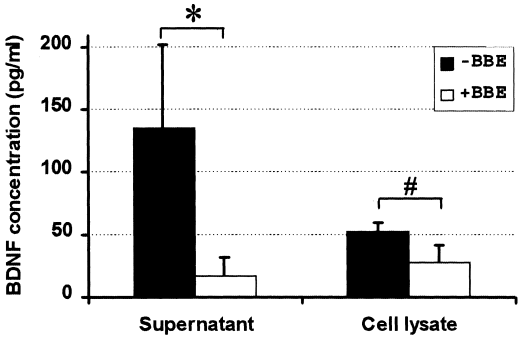

Fig. 1. BDNF synthesis and secretion by cultured HUVECs. HUVECs were cultured in the presence of added BBE until confluency, and the culture medium was replenished without or with added BBE. The cells were then washed three times with PBS. The culture medium was collected after 48 h incubation and the cells were lysed (see Section 2). BDNF in the culture medium and HUVEC cell lysates was measured by ELISA. *$P < 0.01$; #$P < 0.05$.

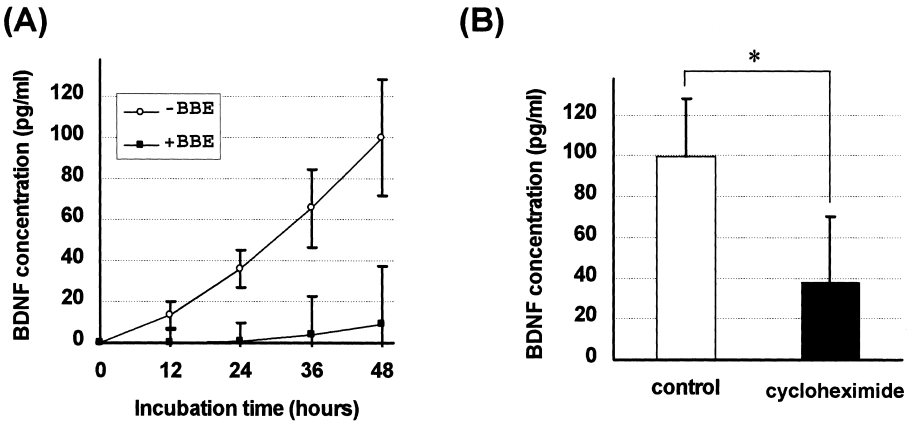

Fig. 2. A: Time course of BDNF production by HUVECs. The cell culture medium without or with BBE was collected at the time indicated. BDNF in the medium was measured by ELISA. B: HUVECs were cultured for 48 h in the medium deficient of BBE, in the absence (control) or presence of CHX (1.0 ng/ml). BDNF in the culture medium was assayed by ELISA. $*P < 0.01$.

HUVECs was constitutively released into the medium. As shown in Fig. 2A, BDNF concentration in the culture medium increased with time whether in the absence or presence of BBE. To test whether the BDNF released by HUVECs was newly synthesized or from a stored pool, CHX (1 ng/ml) was added to the culture medium to inhibit peptide synthesis. After 48 h of incubation, significantly less BDNF (38% of control, $n = 3$) was detected in the medium of cells treated with CHX (Fig. 2B, $P < 0.05$).

3.2. BDNF mRNA in endothelial cells

The expression of BDNF in HUVECs was further confirmed using RT-PCR and Northern blot analysis (Fig. 3). A band at the expected size of 594 bp was amplified with specific primers from the cDNA of HUVECs, HCASMCs and human platelets. The specificity of the band was further confirmed by DNA sequencing. Using Northern blot analysis and a specific probe for BDNF, two strong bands (4.0 and 1.6 kb) were detected in HUVECs and HCASMCs, the same size bands observed in CNS tissue [21]. In Meg-01 and K562 cells that possess megakaryoblastic properties, the two bands were barely detected and appeared very faint. No band was identified in human platelets, suggesting a very low expression level of BDNF in the platelets.

3.3. Regulation of BDNF production

An elevation of intracellular cAMP in HUVECs was achieved with forskolin (1 or 10 μM), which activates adenylyl cyclase directly. This enhanced the BDNF level in the cell culture medium by 2.2- and 2.5-fold, respectively ($P < 0.01$), as detected by ELISA (Fig. 4). This observation was confirmed by adding 500 μM 8-bromo-cAMP, a membrane-permeable cAMP analogue, which increased BDNF by 2.6-fold ($P < 0.01$). The increased release of BDNF by cAMP corresponded with the enhanced BDNF gene expression shown in Fig. 5. Forskolin increased BDNF mRNA even in the presence of BBE (4.1-fold, $P < 0.005$, Fig. 4). Neither IL-1β (10 ng/ml) nor TGFβ (10 ng/ml) affected BDNF expression. On the other hand, addition of the calcium ionophore A23187 (1 μM) to increase intracellular Ca^{2+} reduced BDNF production by HUVEC, to only 2% of the control level obtained without BBE ($P < 0.005$, Fig. 4). Thrombin (1 and 4 U/ml)

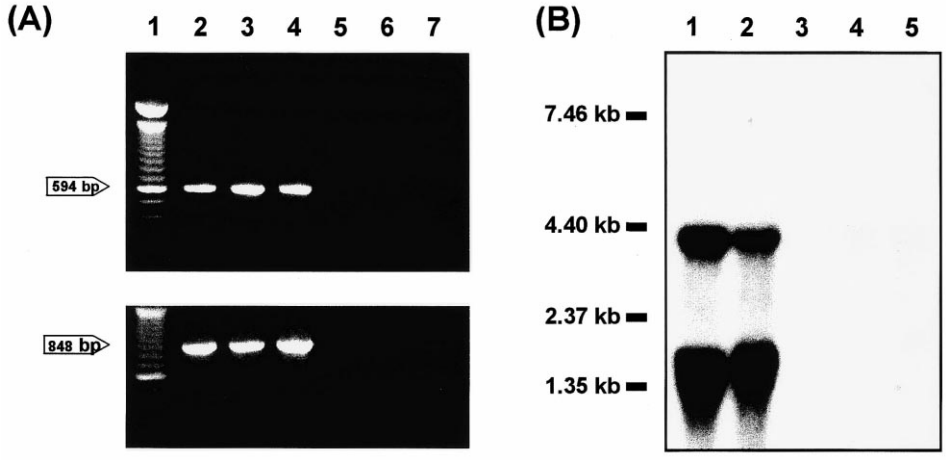

Fig. 3. Gene expression of BDNF in HUVECs. A: Detection of BDNF expression by RT-PCR. Equal amplification of GAPDH in each sample is shown in the lower panel. Lane 1, 100 bp DNA ladder; lane 2, HUVECs; lane 3, HCASMCs and lane 4, human platelets. Lanes 5, 6 and 7 were negative controls (using pseudo RT for PCR) of lanes 2, 3 and 4, respectively. Pseudo RT products (namely RT without adding reverse transcriptase) were used with PCR, and did not identify genomic DNA contamination. B: Northern blot analysis of BDNF expression. Lane 1, HCASMCs; lane 2, HUVECs; lane 3, human platelets; lane 4, Meg-01 cells and lane 5, K562 cells.

and histamine (10 μM), both of which are known to mediate a transient Ca^{2+} increase in HUVECs, did not affect BDNF expression (data not shown). As noted previously (Figs. 1 and 2A), the addition of BBE in cell culture also reduced BDNF release from HUVECs to ~15% of the control without BBE ($P < 0.001$, Fig. 4) and inhibited BDNF gene expression (Fig. 5). Addition of exogenous BDNF (200 pg/ml) to the culture medium deficient in BBE, or the addition of BDNF neutralizing polyclonal antibodies (20 μg/ml) to the medium containing BBE, did not affect BDNF gene expression in the HUVECs (Fig. 5).

3.4. BDNF expression under laminar shear stress

The expression of many genes in HUVECs is regulated by physiological levels of shear stress as reviewed by Davies [22]. Compared to control (stationary) cells, the release of BDNF into the culture medium was reduced with 24 h of shear stress loading to 62% of control ($P < 0.05$, $n = 10$, data not shown).

4. Discussion

BDNF gene expression has been reported in non-nervous tissues including human platelets [10], vascular smooth muscle cells [14] and activated lymphocytes and monocytes [17]. In the present study, the expression of BDNF was also demonstrated in vascular endothelial cells. Our results implicate BDNF mRNA and protein release for the ability of endothelial cells to promote neuron growth in co-culture [23]. In this report, however, the BDNF level in cell culture medium measured by ELISA was nearly 3-fold higher than our values. The difference may be explained by the different ELISA kit (Promega vs. Regeneron) used for BDNF measurement and the different media used for the cell culture procedures. It was also observed that the passage number and HUVEC seeding density also affected BDNF expression. As the passage number increased, less and less BDNF was detected in the culture medium.

The BBE used in our culture medium dramatically inhibited BDNF expression (by ~60%, Fig. 5B). This suppression is not due to feedback inhibition by BDNF that is probably contained in the BBE, since adding exogenous BDNF did not affect BDNF gene expression by HUVECs. Instead, these

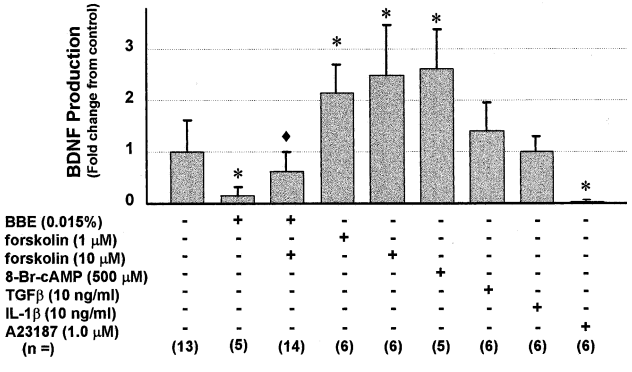

Fig. 4. Regulation of BDNF production in HUVECs. HUVECs were cultured with the indicated stimuli for 48 h, and BDNF in the culture medium was measured by ELISA. BDNF concentration in the culture medium without BBE was used to normalize the data. Bars show the fold increase from control and error bars show S.D.s. $^{*}P < 0.01$ vs. control; $^{\blacklozenge}P < 0.05$ vs. +BBE.

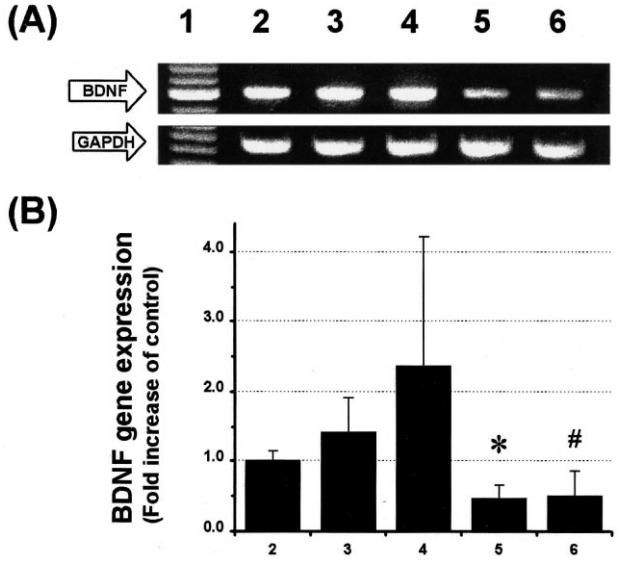

Fig. 5. Regulation of BDNF gene expression in HUVECs detected by RT-PCR. (A) BDNF and GAPDH expression in HUVECs. HUVECs were harvested for total RNA extraction (and subsequent RT-PCR) after 24 h culture in the medium deficient of BBE (lane 2), plus 200 pg/ml exogenous BDNF (lane 3), or plus 10 mM forskolin (lane 4), or in the medium with BBE (lane 5), or with BBE and 20 μg/ml polyclonal neutralizing antibodies against BDNF (lane 6). Lane 1, DNA ladder. (B) Densitometry of PCR products from (A). The data were expressed as ratio of BDNF/GAPDH. The value for the cells cultured in BBE deficient medium was taken as one for normalization. $n = 4$, $^{*}P < 0.01$; $^{\#}P < 0.05$.

results suggest that there may be endogenous inhibitor(s) for BDNF expression in the CNS, which may regulate BDNF expression in vivo, and warrant further investigation.

As demonstrated by Northern blot analysis and RT-PCR, the expression levels of BDNF in human platelets, Meg-01 cells and K562 cells were very low. Almost no BDNF protein was detected in the cell culture medium or in cell lysates of Meg-01 and K562, whereas the BDNF content of human platelets or that released after platelet activation is in the range of multiple ng/10^8 platelets [11]. Together, our results support the hypothesis that most of the BDNF present in platelets is sequestered from blood, and that vascular endothelial cells may be one of the major sources of BDNF in platelets and thus in circulation.

The BDNF expression of neuronal and neuron support cells has been shown to be regulated by cAMP. In cultured rat Schwann cells, forskolin or 8-bromo-cAMP suppress BDNF expression [24]. However, elevations of cAMP increase BDNF expression in neurons of the rat frontal cortex and hippocampus [25]. The cAMP responsive element binding protein has been implicated in the up-regulation of BDNF expression [26]. DNA sequence analysis identified a putative cAMP responsive element (5′-TGACTTCA-3′) in the promoter region of the BDNF gene (at −1682). A similar up-regulation of HUVEC BDNF expression by forskolin or 8-bromo-cAMP was observed in the present study. It is known that elevation of intracellular Ca^{2+} in HUVECs increases the release of many substances including nitric oxide and prostacyclin (PGI_2). Our results showed that calcium ionophore inhibited BDNF production, whereas thrombin or histamine had no effect on BDNF generation in HUVECs. DNA sequence analysis of

the BDNF gene also indicates the presence of a shear stress responsive element (5′-GAGACC-3′) [27] in the promoter region (at −421) and in the first intron (at +595 and +629). Indeed, exposure of HUVECs to physiological levels of shear stress down-regulated BDNF expression, similar to that seen with endothelin [28]. Although the physiological significance of the down-regulation by shear stress is unclear, changes in shear stress have been reported to have a close relation to atherosclerosis. It was observed that atherosclerosis occurred at the low shear stress area in the aorta [29].

The cardiovascular role of BDNF is unknown. BDNF functions through a specific high-affinity receptor of tropomyosin receptor-related kinase (Trk) B, which is a transmembrane tyrosine kinase. The expression of TrkB has been reported in cultured rat and human vascular smooth muscle cells [14,30]. TrkB expression in smooth muscle cells is found in human coronary atherosclerotic lesions, and increases dramatically in the area of injury and persists during the formation of the neointima in a rat aortic balloon de-endothelialization model [14]. Only truncated TrkB which lacks the tyrosine kinase domain was detected in HUVECs by RT-PCR (unpublished observations). It is interesting to note that platelets release large amounts of BDNF rapidly upon activation by thrombin, calcium ionophore or collagen [11], which probably occurs at the site of vascular injury as well. BDNF released in this manner or released from endothelial cells could bind to TrkB on smooth muscle cells or endothelial cells, but the functional effects of the binding remain to be determined. Our data suggest that vascular endothelial cells are a source of circulating BDNF in the blood.

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